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# How a leaf gets its shape

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Leaves are formed from a group of initial cells within the meristem. One of the earliest markers of leaf initiation is the down-regulation of *KNOX* genes in initial cells. Polar auxin activity, MYB and LOB domain transcription factors function to keep *KNOX* out of the initiating leaf. If *KNOX* genes are expressed in initial cells, leaves fail to form. As the leaf grows away from the meristem, its shape is determined by growth in three axes, proximal–distal, abaxial–adaxial and medial–lateral. HD-ZIPIII, KANADI and the small RNA pathway play a significant role in the latter two axes. KNOX proteins play a role in the proximal–distal axis. Although genetic networks are conserved between monocots and dicots, the outcome in leaf shape often differs.

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## Introduction

Coordination of cell division and differentiation is required for the development of multicellular organisms, which start as single cell zygotes. In plants, organs are continuously generated at the flanks of the meristem, where a population of pluripotent stem cells resides. At the center of the dome-shaped shoot apical meristem (SAM), cells divide slowly to generate more cells that will be used for lateral organs. When stem cells divide, daughter cells are shifted towards the periphery where organ initiation and/or further division occur. Cell fate is therefore connected to a cell's location within the meristematic dome.

During vegetative growth, the SAM continues to produce leaves, which initiate as dorsiventral structures from the flank of the meristem. Leaves capture light energy and use it to convert carbon dioxide and water into sugars and oxygen that will be the energy source for the whole plant. Thus, a leaf needs an optimal structure to maximize light capture and be efficient in gas exchange. At the same time, leaves have evolved mechanisms, such as dissection, to protect themselves from excessive damage from insects, wind or sun. While leaves are often large and serve as the major photosynthetic structures of plants during vegetative growth, as the plant becomes reproductive, the leaves become small and subtend flowers or branches. The process of leaf development, from initiation to patterning, involves coordinated regulation among transcription factors, small RNAs and hormones. In this review, we will discuss the mechanisms underlying the transition to determinancy during leaf initiation and how polarity is maintained during leaf outgrowth.

## Leaf initiation: a switch to determinancy

The pluripotent state of the SAM is characterized by expression of Class I *KNOTTED1-LIKE HOMEOBOX* (*KNOX*) genes [1,2]. Loss-of-function mutants of the *KNOX* genes, *knotted1* (*kn1*) in maize and *SHOOTMERISTEMLESS* (*STM*) in Arabidopsis, show loss of meristem function to various degrees indicating that these genes are required for proper maintenance of the SAM [3,4]. One of the earliest indications of leaf development is the down-regulation of *KNOX* genes at the site of incipient primordia. Through this repression, a switch from an indeterminate to a determinate fate occurs in the small group of cells that will be the immediate precursor of the leaf primordium. KNOX transcription factors regulate two phytohormones, gibberellin (GA) and cytokinin. GA promotes cell elongation and differentiation while cytokinin promotes cell proliferation within the meristem. Thus, a high cytokinin to low GA ratio is important to maintain the SAM in an indeterminate state. KNOX proteins directly downregulate the *GA20-oxidase* gene, an enzyme encoding a rate-limiting step in GA biosynthesis [5,6]. Inactivation of GA is also achieved through accumulation of the *GA2-oxidase* by KNOX proteins [7•]. On the other hand, KNOX proteins upregulate the expression of cytokinin biosynthesis genes to achieve high levels of cytokinin [8,9]. The downregulation of *KNOX* genes from the incipient primordium will lead to a low cytokinin to high GA ratio promoting the switch from an indeterminate to a determinate state.

Polar auxin transport is considered important for the downregulation of *KNOX* genes in the incipient primordium in both maize and Arabidopsis. Maize apices grown in the presence of an auxin transport inhibitor fail to show *KNOX* downregulation within the meristem and fail to initiate leaves [10]. The *bobber1* mutant of Arabidopsis, which is blocked at the globular stage of development, expresses *STM* throughout the apical half of the embryo

and auxin activity, as visualized by DR5, is not localized [11•]. In addition, real-time imaging of developing apices showed localized auxin activity correlates with the site of *KNOX* downregulation [12].

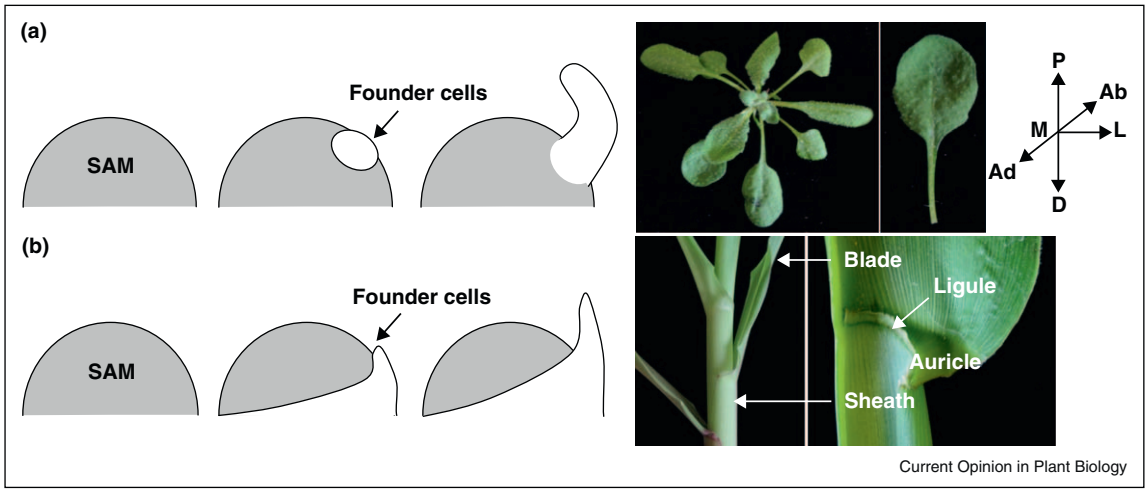
Another pathway involved in *KNOX* repression is mediated by the *ARP* genes, named after *ASYMMETRIC LEAF1* (*AS1*) from Arabidopsis, *rough sheath2* (*rs2*) from maize, and *phantastica* (*phan*) from *Antirrhinum*. These genes encode MYB transcription factors and are expressed in lateral organ founder cells where they repress *KNOX* expression (reviewed in [13]). AS1 forms a repressor complex with the LOB domain protein AS2 that directly binds to specific regions of *KNOX* promoters [14]. The repressor complex also includes the predicted RNA binding protein RIK and the chromatin-remodeling protein HIRA, suggesting formation of a repressed chromatin state at the targeted *KNOX* locus during organogenesis [14,15]. In addition, genetic experiments suggest that AS1 function converges with auxin to repress the *KNOX* gene *BREVIPEDICELLUS* (*BP*) [16]. Thus, multiple levels of regulation and many partners are likely to keep *KNOX* genes out of the leaf initiation site.

## Asymmetric growth of the leaf: which side is up?

Starting as a bulge on the flank of the SAM, the newly initiated leaf becomes asymmetric in several axes: the adaxial–abaxial, medial–lateral, and proximal–distal (Figure 1). The meristem provides leaves with an inherent sidedness, with the adaxial side adjacent to the meristem and the proximal end attached to the meristem. Polarization along these axes eventually leads to the asymmetric distribution of cell types in the mature leaf that is critical for the physiology of the plant. For example, the adaxial side of the leaf blade often consists of cells specialized for light harvesting while the abaxial side contains cells involved in gas exchange to maximize photosynthesis. Similarly, the distal end of a pea leaf contains the tendrils that cling to support structures and the proximal end of a grass leaf is the sheath, specialized for holding the leaf to the stem.

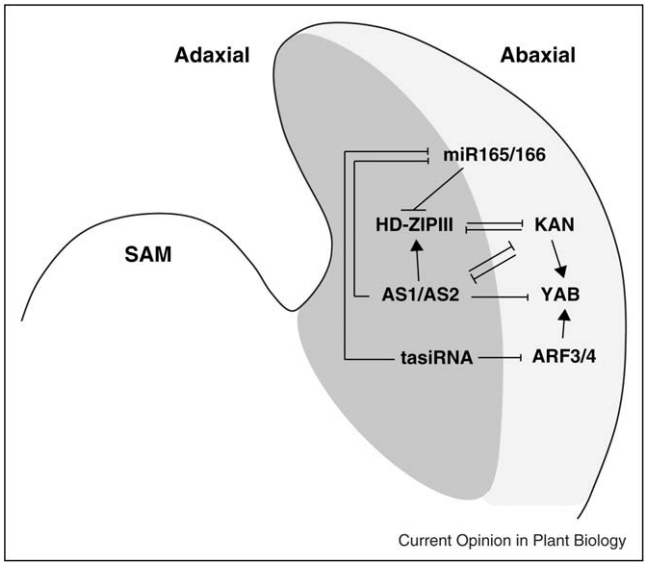
Characterization of polarity mutants led to the discovery of a complex regulatory network underlying the process of polarity establishment and leaf outgrowth. Key to this discovery was analysis of the dominant mutants, *phavoluta* (*phv*), *revoluta* (*rev*) and *phabulosa* (*phb*) in Arabidopsis and *Rolled1* (*Rld1*) in maize [17–19]. The genes defined by these mutants encode class III HOMEODOMAIN LEUCINE ZIPPER (HD-ZIPIII) proteins [18,20,21]. Expression of this gene family is restricted to the adaxial side of the developing leaf and extends into the SAM in a pattern that predicts phyllotaxy [18]. In mutants lacking HD-ZIPIII function, abaxial fate dominates and the SAM fails to form. Gain-of-function mutants occur due to mutations in a miR165/166 binding site within the gene preventing the transcripts from being cleaved [22,23]. In non-mutant leaves, miR165/166 is expressed towards the abaxial side limiting the expression of *HD-ZIPIII* to the adaxial domain of the leaf (Figure 2). In the dominant mutants, *HD-ZIPIII* expression spreads throughout the leaf resulting in adaxialization.

**Figure 1**




Leaf initiation in **(a)** Arabidopsis and **(b)** maize. Class I *KNOX* genes are expressed throughout the SAM keeping the cells in an indeterminate state [1,2]. At the site of incipient primordia, downregulation of *KNOX* genes occurs which leads to a switch from an indeterminate to a determinate fate in the small group of cells (founder cells) that will be the immediate precursor of the leaf primordium. The developing leaf becomes asymmetric in several axes: proximal–distal, adaxial–abaxial and medial–lateral. This polarization leads to asymmetric distribution of cell types in the mature leaf. In maize, a leaf develops in a ring-like structure where the proximal end will differentiate into sheath tissue which wraps around the stem. *KNOX* expression domain is colored in grey. P: proximal, D: distal, M: medial, L: lateral, Ad: adaxial, and Ab: abaxial.

**Figure 2**




Establishing adaxial–abaxial polarity in a developing leaf. *HD-ZIPIII* is expressed in the adaxial domain [18] while *KAN* sets up the abaxial domain of the leaf [24,25]. The antagonistic relationship of these two gene families is established by several layers of regulation. miR165/166 negatively regulates *HD-ZIPIII* to prevent expression on the abaxial side of the leaf [22,23] while the AS1/AS2 complex promotes the expression of *HD-ZIPIII* in the adaxial domain [37]. The AS1/AS2 complex also represses the expression of miR165/166, *KAN* and *YAB* [37,38]. The *ARF3/4* transcription factors promote the abaxial fate and the tasiRNAs prevent these genes from being expressed adaxially [47,48••].

Antagonistic to the *HD-ZIPIII* genes are also the *KANADI* (*KAN*) gene family members, expressed on the abaxial side of the leaf primordia [24,25] (Figure 2). Loss-of-function *kan* mutants are adaxialized and *HD-ZIPIII* genes are expressed throughout the leaf whereas *KAN* overexpression causes abaxialization. The *YABBY* (*YAB*) genes function relatively later in leaf development and are considered to act downstream of the *KAN* genes. In Arabidopsis, *YAB* genes expression is abaxial and adaxialization occurs when multiple *yab* mutations are combined [26,27•].

Three recent reports highlight *kan* loss-of-function mutants in maize and rice. The rice mutant, *shallot-like1* (*sll1*) has rolled leaves due to loss of schlerenchymatous cells on the abaxial sides of the veins [28•]. Ligules, normally only adaxial, are also found abaxial. The *milkweed pod1* (*mwp1*) mutant of maize affects the sheaths of leaves in patches and not the blade, although the gene is expressed in both sheath and blade [29•]. The *HD-ZIPIII* gene *Rld1* is misexpressed in these patches. Sheaths are narrower as are leaf-like organs of the flower [30•]. Prophylls, which are made by the fusion of the first two leaves of the axillary branch, are thread-like and unfused. The narrow leaves in *mwp1* mutants support the model of Waites and Hudson that proper abaxial–adaxial patterning is required for lamina outgrowth [31].

Genes that were identified as negative regulators of *KNOX* genes are also implicated in polarity. The first polarity mutant characterized was the Antirrhinum *phan*, which has a mutation in a gene orthologous to *AS1* in Arabidopsis [31,32]. Leaves of the *phan* mutant display abaxialization. Mutations in the homologous genes Arabidopsis *AS1* and maize *rs2*, rarely have defects in leaf polarity [33–35]; however, AS1 plays a role in abaxial–adaxial polarity formation by forming a complex with AS2 [36–38]. The AS1/AS2 complex positively regulates *HD-ZIPIII* expression and suppresses *KAN* and *YAB* expression [37] (Figure 2). The positive regulation of *HD-ZIPIII* by the AS1/AS2 complex is also achieved through reducing miR165/166 expression [38]. A direct interaction of AS2 and KAN was shown through a novel dominant allele. *as2-5d* has polarity defects that resemble *kan* mutants [39]. KAN directly binds to the *AS2* promoter but does not bind to the promoter in the dominant mutant, thereby resulting in high *AS2* levels. The high levels of *AS2* in turn, directly or indirectly, negatively regulate *KAN*. These results may explain why *phan*, which is not expressed in a polar fashion, has a polarity defect. Perhaps the loss of *phan* leads to an increase in *KAN* and thus abaxialization.

Other polarity determinants, the auxin response factors ARF3/ETTIN and ARF4, were identified as suppressors of a *KAN* overexpressing plant [40]. They function downstream of *KAN* but additional regulation is achieved by trans-acting short interfering RNAs (tasiRNAs) [41,42]. TasiRNAs are generated from *TAS* genes, which do not encode a protein but function as an RNA. The production of tasiRNAs requires a number of proteins including AGO7, SGS3, RDR6 and DCL4 [43–46]. *ARF3* and *ARF4* mRNAs are cleaved and degraded by these tasiRNAs [47]. tasiRNAs targeting the ARFs are produced at the adaxial side of the primordium and prevent ARF activity in the adaxial domain [48••] (Figure 2). The two small RNAs, tasiRNA and miR165/166, show opposite polar distribution in leaf primordia and are thought to establish the adaxial–abaxial axis in leaf development [42].

Mutations in the tasiRNA biogenesis pathway or plants with TAS3-insensitive *ARF3* do not show striking polarity defects in Arabidopsis [47,49], but they do in the grasses. In rice, mutants in *RDR6* (*shootless2*), *AGO7* (*shootless4*) and *DCL4* (*shoot organization2*) produce shootless embryos or filamentous leaves [50,51]. In maize, the *leafbladeless1* (*lbl1*) mutant, which carries a mutation in *SGS3*, shows radialized abaxialization [42]. The maize *ago7* mutant, which was recently shown to be *ragged seedling2* (*rgd2*) [52••], has cylindrical leaves, but surprisingly, no loss of dorsiventrality [53]. *rgd2-r* mutants have less tasiARF and

an increase in *ARF3A*, but expression of *ARF3A* is still polarized in both *rgd2* and in *lbl1* mutants [52••]. The difference in polarity between *lbl1* and *rgd2* mutants suggests that LBL1 does more than accumulate tasiRNAs. The regulation of leaf polarity is likely through *AGO1*, which is increased in *lbl1* but not *rgd2* mutants [52••] and accumulates adaxially in maize [54].

## The role of *KNOX* genes in proximal distal polarity and leaf complexity

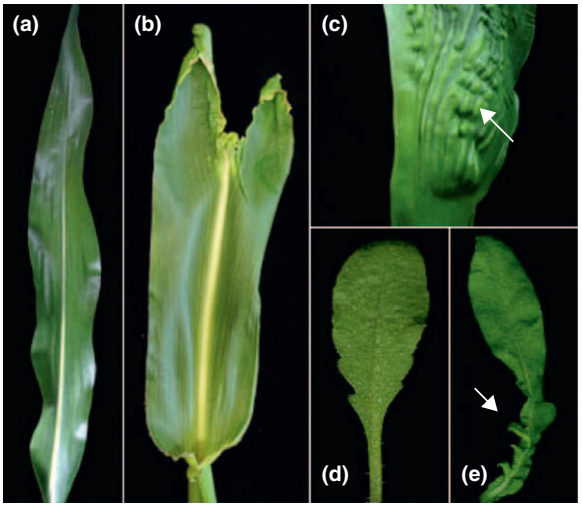
Just as the absence of *KNOX* genes plays a role in defining the position of a leaf within the meristem, expression of *KNOX* genes plays a critical role in elaborating leaf shape in many species (reviewed in [55,56]). The timing and location of *KNOX* expression in the leaf turns out to be critical for the outcome.

Maize leaves have a proximal sheath that wraps tightly around the stem and a distal blade that lies back to optimize photosynthesis. At the junction of the sheath and blade is a ligule region that contains an adaxial ligule fringe and two auricles (Figure 1). Maize, like other plants with simple leaves, does not express *KNOX* genes in the leaf; however, a number of dominant mutants exist in which *KNOX* genes are expressed in the leaf [57,58]. For most of these mutants, *Liguleless3*, *Roughsheath1*, *Gnarley*, and some *Kn1* alleles, the ligule region is displaced to a more distal position. In cases where this has been studied by *in situs*, *KNOX* expression extends into the sheath from the adjoining stem [59,60]. In some *Kn1* alleles, however, the misexpression is not contiguous with expression in the meristem, but is in the veins of the blade or along the margins. The outcomes are quite different depending on when and where expression occurs (Figure 3). When *kn1* is expressed in the lateral veins, the cells adopt sheath and auricle fates and form protrusions known as knots [61]. However, when *kn1* is expressed at the margin, flaps of tissue grow out from the margin that are sheath and auricle in leaf cell type. When *kn1* is expressed at the very tip of the growing leaf, growth is arrested and the leaf becomes bifurcated [62•]. These phenotypes suggest that *kn1* regulates proximal distal patterning [62•].

Another mutant with defects in proximal distal identity is *blade on petiole* (*bop*) of Arabidopsis. Ectopic blade tissue is found in a proximal position, along the petiole (Figure 3) [63,64]. *bop* mutants accumulate *KNOX* genes in the leaf and the BOP1/BOP2 complex directly and positively regulates AS2 [65•]. Thus, the absence of BOP function leads to less AS2 and more KNOX, yet the phenotype, a proximal (petiole) to distal (blade) transformation, is opposite to that of the maize mutants [57].

Tomato leaves have a terminal leaflet and alternating leaflets from the main rachis. Second order leaflets occur from the first order leaflets, but not from the terminal leaflet. *KNOX* genes are expressed in leaves and

**Figure 3**




Ectopic *KNOX* expression in leaves leads to a different outcome depending on the location and timing. In normal maize, *kn1* expression is excluded from the developing leaf. The *Kn1-DL* and the *Kn1-O* alleles are shown here which have ectopic *kn1* expression in the leaves [61,62•]. In the *Kn1-DL* allele, *kn1* is expressed at the very tip of the growing leaf leading to arrest in growth and the leaf bifurcation **(b)**. In the *Kn1-O* alleles, the ectopic *kn1* expression results in knots (shown with an arrow) which consists of cells with sheath and auricle fates **(c)**. *KNOX* expression is also excluded from the developing leaf of Arabidopsis. When *KNOX* genes are turned on in leaves, as in the case of *bop* mutants, ectopic blade tissue appears at a proximal position, along the petioles (indicated with an arrow) **(d,e)** [63,64].

additional expression often leads to further dissection of the first order leaflets (reviewed in [56]). Shani *et al.* expressed the STM ortholog of tomato, TKn2, behind the FIL promoter, which gives expression in young leaf primordia but not the meristem [66]. Constructs either included or did not include an EAR repressor domain [67••]. Surprisingly, both constructs led to simpler leaves, but for different reasons. Plants that expressed *FIL:TKn2-EAR* prematurely differentiated, before developing leaflets, whereas expression of *FIL:TKn2* kept leaves in a perpetual early plastochron state, unable to expand the lamina. They also used promoters that function at different times of leaf maturation. Only promoters that function during the primary morphogenesis state increased the complexity of the leaf [67••].

One of the facilitators of leaf diversity generated by KNOX is likely to be the *CUP-SHAPED COTYLEDON* (*CUC*) gene family, which is expressed at the boundary of leaves and leaflets [68,69]. Dex induction of a KN1-GR fusion led to an increase in lobing and an increase in *CUC* gene expression in *Cardamine* [70]. Loss of *CUC* gene function led to a less dissected leaf in a number of species including pea, potato, tomato, *Cardamine* and *Aquilegia*

[70]. However, overexpression of a microRNA resistant version of the tomato *CUC* gene, *GOBLET* also had fewer leaflets, presumably because of leaflet fusion [71]. Clearly the context of both *KNOX* and *CUC* expression is critical for the genesis of leaf shape, whether they make a leaf dissected or simple, and whether they displace proximal or distal tissues.

## Conclusion

Diverse species often utilize the same set of regulators to elaborate structures. Slight changes in timing and expression pattern of these regulators are likely to result in dramatic morphological variation. Plant scientists are in position to take advantage of the knowledge gained from model organisms to begin to probe evolutionary change, and to move from the transcription factor regulators to understanding the cellular processes of growth.

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